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Proton translocation by cytochrome *c* oxidase in different phases of the catalytic cycle

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Abstract

Since mitochondrial cytochrome *c* oxidase was found to be a redox-linked proton pump, most enzymes of the haem-copper oxidase family have been shown to share this function. Here, the most recent knowledge of how the individual reactions of the enzyme's catalytic cycle are coupled to proton translocation is reviewed. Two protons each are pumped during the oxidative and reductive halves of the cycle, respectively. An apparent controversy that concerns proton translocation during the reductive half is resolved. If the oxidised enzyme is allowed to relax in the absence of reductant, the binuclear haem-copper centre attains a state that lies outside the main catalytic cycle. Reduction of this form of the enzyme is not linked to proton translocation, but is necessary for a return to the main cycle. This phenomenon might be related to the previously described "pulsed" vs. "resting" and "fast" vs."slow" forms of haem-copper oxidases.

Keywords: Proton translocation; Cytochrome *c* oxidase; Catalytic cycle

1. Introduction

Cytochrome *c* oxidase is a redox-linked proton pump, the turnover of which creates protonmotive force across the mitochondrial or bacterial membrane with an efficiency of two translocated charges per transferred electron in the steady state [1]. This function is shared among the various prokaryotic haem-copper oxidases, including the quinol oxidase family of enzymes [2,3]. The mechanism of redox-linked proton pumping has remained elusive, however, despite the availability of X-ray structures of several haem-copper oxidases (see Refs. [4–6]). The research has recently comprised kinetic studies of individual reaction steps in the catalytic cycle and their linkage to proton transfer processes, and has included time-resolved electrometric experiments on enzyme incorporated into phospholipid vesicles.

2. Intermediates of the catalytic cycle

Fig. 1 depicts major intermediate states of the enzyme's binuclear haem a_3/Cu_B site during catalysis. The cycle may a priori be divided into an oxidative (intermediates **R** to **O**) and a reductive phase (intermediates **O** to **R**). Fig. 1b shows the corresponding tentative structures of the binuclear site. As will become apparent below, this description of the cycle is too simplistic, as it does not account for results from recent experiments.

3. Proton pumping in the catalytic cycle

A current controversy relates to the question of which steps in the cycle are linked kinetically to proton translocation. This is an important issue that must be resolved before the mechanism of proton translocation can be addressed seriously. Direct measurements of proton translocation during a single turnover, initiated by adding O_2 to the fully reduced enzyme, revealed pumping of two protons during the oxidative phase of the catalytic cycle (one proton pumped each in the **P** → **F** and **F** → **O** steps), and pumping

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of two more protons during the reductive phase [7]. This finding was surprising in relation to the earlier view that all proton translocation would take place in conjunction with the oxidative phase, which was based on equilibrium titrations in intact mitochondria at high protonmotive force [8]. This earlier view was also supported by the fact that the **P** → **F** and **F** → **O** reaction steps may be expected to be strongly driven thermodynamically especially when compared to the known relatively low midpoint redox potentials involved in the reductive half of the cycle ([9]; Fig. 1b, cf. below).

Another surprising finding was that proton pumping during the reductive phase was observed *only when this phase was allowed to take place immediately after the oxidative phase*. Thus, reduction of the enzyme without an immediately preceding oxidation by O_2 failed to yield proton pumping [7]. These observations were interpreted

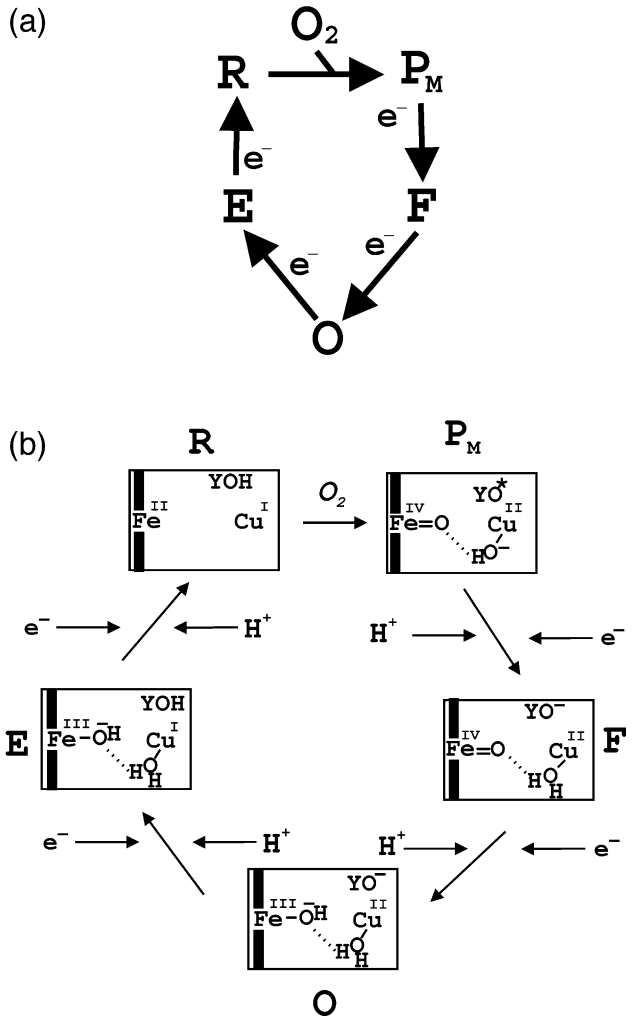

Fig. 1. (a) Simplified scheme of the catalytic cycle of cytochronme *c* oxidase. Intermediate states of the binuclear haem-copper centre are shown (**R**, reduced; $\mathbf{P_M}$, "peroxy"; **F**, ferryl; **O**, oxidised; **E**, one-electron reduced). (b) Corresponding tentative structures of the binuclear centre showing the states of haem a_3 and Cu_B. YOH, YO* and YO^- represent the neutral, radical and anionic forms of a conserved tyrosine residue in the site. Proton translocation is not depicted.

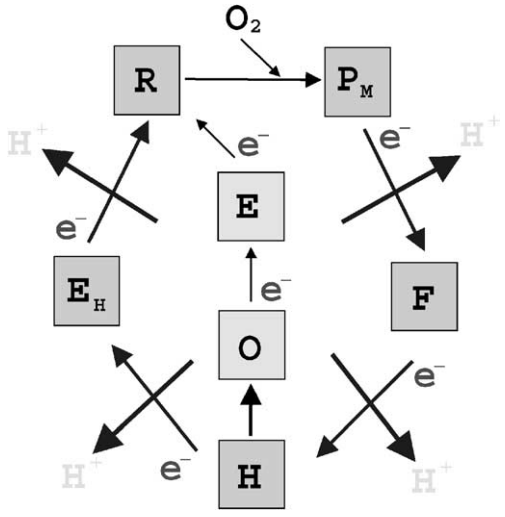

Fig. 2. Current view of the catalytic cycle. The main cycle (in blue) involves the intermediates **H** and $\mathbf{E_H}$ that represent metastable forms of the oxidised and one-electron reduced binuclear site (replacing **O** and **E** in Fig. 1a). In this scheme, each one-electron step in the main cycle is depicted to be linked to translocation of one proton (see the text). In the absence of reductant, the **H** state decays into state **O** outside the main cycle. Reactivation of the enzyme requires reduction of **O** to **R** (via **E**), which is not coupled to proton translocation.

to mean that the immediate product of the oxidative phase is a metastable form of the **O** state (**O**~, here called **H**) that is capable of sustaining proton translocation when triggered by arrival of electrons, but that will otherwise decay to the more stable **O** state.

Fig. 2 summarises this more recent view. Here both reactions of the reductive phase have been tentatively depicted to be linked to proton translocation, but at the present time we do not know for certain how the observed pumping of two protons is distributed during the reductive phase. Fig. 2 is also consistent with the experimental data showing that the oxidative and reductive halves of the catalytic cycle are associated with an approximately equal number of translocated electrical charges [7]. As depicted schematically in Fig. 2, we find it necessary to postulate a main catalytic cycle that involves the hypothetical states **H** and $\mathbf{E_H}$, and which is associated with pumping of four protons [7]. However, in the absence of a sufficient electron donor, the enzyme in state **H** relaxes to a more stable oxidised state (**O**) which lies outside the main cycle. The main cycle may be re-entered by reducing **O** to **R**, but this sequence is not coupled to proton translocation.

4. Electrometric measurements

The electrometric technique [10–12] may be used to monitor charge translocation due to both electron and proton transfer perpendicular to the membrane during different reaction steps in the catalytic cycle. Thus, it is a powerful method for an independent test of whether

reduction of the relaxed oxidised enzyme (in state **O**) is coupled to proton translocation. One practical problem here, however, is to ensure that the state of the enzyme is initially the one anticipated. Ruitenberg et al. [13] reported that photoinjection of the first electron into the relaxed enzyme in state **O** (to generate **E**) is coupled to two electrogenic phases, one fast (ca. 15 μs) due to electron transfer from Cu_A to haem *a*, and one slower phase (ca. 150 μs), which may be attributed to proton uptake. In contrast, we have found that this biphasic response is obtained specifically upon injecting the *second* electron into the enzyme (i.e. linked to the reaction $\mathbf{E} \rightarrow \mathbf{R}$), whereas injection of the *first* electron only yields the fast electrogenic phase due to electron transfer from Cu_A to haem *a*, but no slower phases within tens of milliseconds (Fig. 3; [14]). Another reason for why the slow 150-μs phase cannot be attributed to injection of the first electron is that its amplitude increases with flash number (Fig. 3), while the amplitude of an event associated with injection of the first electron must decrease [14]. The probable cause of this difference in results may be found in the experimental conditions in Ref. [13], where the enzyme was suspended in the presence of ruthenium bispyridyl, ferricyanide, glucose and glucose oxidase. Such conditions are bound to lead to partial reduction of the enzyme before the first laser flash (see Ref. [14]).

We ascribe the second $\tau \sim 150$ μs phase observed during the $\mathbf{E} \rightarrow \mathbf{R}$ transition to proton uptake into the binuclear centre accompanying electron equilibration between haem *a* and that centre when the second electron is injected into the enzyme [12]. The amplitude of this phase is consistent with the redox potentials of haem *a* and Cu_B, and with the finding that these centres lie 32% into the membrane dielectric from the positively charged *P*-side [7,12]. Michel et al. [13,15] agree with us [7] that there is no proton translocation on injection of the first electron into the relaxed oxidised enzyme, but they propose that proton translocation plus net proton uptake is associated with the second electron. However, the amplitude of the 150-μs phase is far too small to be consistent with this latter proposal.

Thus, based on both direct proton measurements and electrometric data, we conclude that no proton pumping is associated with the reduction of the relaxed oxidised enzyme, i.e. during neither of the reactions $\mathbf{O} \rightarrow \mathbf{E}$ and $\mathbf{E} \rightarrow \mathbf{R}$.

Recently, Ruitenberg et al. [16] reported new electrometric observations of proton pumping during reduction of cytochrome *c* oxidase by a second electron (step $\mathbf{E} \rightarrow \mathbf{R}$). In this work the intermediate **E** was generated chemically from the **F** state (formed with H_2O_2) using carbon monoxide, which is known to reduce the ferryl haem in **F** by two electrons to yield ferrous haem plus CO_2 [17]. Photoinjection of an electron into the **E** state thus generated yielded a millisecond electrometric response that was interpreted to be due to proton pumping. The authors contrasted this result to our previous report that a preceding oxidation of reduced enzyme with O_2 was necessary in order to observe proton-pumping during the reductive phase [7]. However, in our view these results in fact provide further evidence for the notion that as long as the enzyme is not allowed to exit from the main catalytic cycle via dissipation of the **H** state to **O**, the reactions of the reductive phase are coupled to proton translocation. Thus, we expect that in these experiments the catalytic cycle has been short-circuited by strict two-electron reduction of the **F** state to $\mathbf{E_H}$ (Fig. 2). By such a procedure the enzyme is not expected to depart from the main catalytic cycle since the **H** state has been avoided.

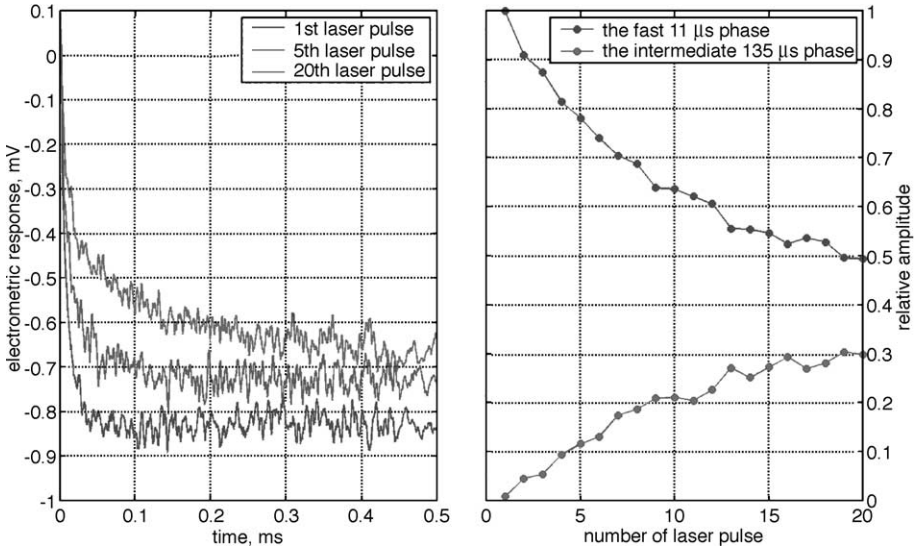

Fig. 3. Kinetics of electric potential generation in wild-type cytochrome *c* oxidase upon flash-induced electron transfer into oxidised enzyme from *P. denitrificans* (left panel). The right panel depicts the dependence of the amplitudes of the fast and slow electrometric phases on the pulse number. For experimental conditions, see Ref. [14].

5. Some thermodynamic considerations

Proton translocation coupled to the ''side track'' sequence of events $\mathbf{O} \rightarrow \mathbf{E} \rightarrow \mathbf{R}$ is contradicted also on thermodynamic grounds. The measured equilibrium $E_{m,7}$ values for haem a_3 and Cu_B are at most 0.4 V in these transitions (see Ref. [9]), and the electron donor, cytochrome *c*, operates at a potential of ca. 0.3 V in aerobic steady states. The available driving force, ca. 100 mV, is far too small to drive proton translocation against a protonmotive force of at least 150 mV, especially bearing in mind that charge is translocated not only due to proton-pumping, but in addition due to electron transfer into the binuclear site and proton uptake to form water from reduced O_2. Thus, at a protonmotive force of 150 mV, the driving force on the reductive steps, if linked to proton-pumping, must be higher than 300 mV to produce significant flux.

Two counter-arguments may be raised against this conclusion. The first is that the operating potentials of the **E**/**R** and **O**/**E** redox couples may be much higher than the measured $E_{m,7}$ values if the **E**/**R** and **O**/**E** ratios are very high in the steady state. This argument is invalidated by the finding of Gnaiger et al. [18] that the efficiency of oxidative phosphorylation is unimpaired, and actually increased at O_2 concentrations below the apparent K_M. Under such conditions the steady state occupancy of the **R** state must be considerable. Interestingly, under such conditions the occupancy of the metastable **H** state would also be expected to be low.

The second counter-argument is that proton-pumping is normally measured at ''level flow'' conditions, i.e. with no opposing protonmotive force. In such conditions, a low driving force on the redox reactions in the reductive half of the catalytic cycle could easily suffice to drive proton translocation. At high physiological protonmotive force proton-translocation might simply not take place during these partial reactions, which would lead to a drop in the efficiency of ATP synthesis. Again, this is contradicted by the findings discussed above [18], but another independent analysis is even more revealing: 10 protons are translocated across the F_o segment of ATP synthase per revolution in eukaryotic mitochondria [19], and 3 ATP molecules are synthesised. This means that 3.33 H^+ are translocated across F_o per ATP produced intramitochondrially. One additional proton needs to be translocated to export the ATP into the cytosol (including import of ADP and P_i), yielding 4.33 translocated H^+ ions per produced extramitochiondrial ATP. Chamalaun and Tager [20] found in careful measurements that 0.94 ± 0.02 ATP was produced per oxygen atom consumed when phosphorylation was linked to the cytochrome *c* oxidase reaction in mitochondria. According to this, cytochrome *c* oxidase must translocate 0.94×4.33 or 4.07 charges per consumed oxygen atom to produce this amount of ATP, i.e. 2.0 charges per electron, which is precisely the known charge translocation stoichiometry of the enzyme as measured in the absence of protonmotive force [1,9]. Hence, there is no measurable ''slipping'' in the coupling between the redox reaction and proton pumping in cytochrome *c* oxidase due to high protonmotive force, which is at least 150 mV during net ATP synthesis [21].

It follows, not only that proton-pumping cannot occur in the $\mathbf{O} \rightarrow \mathbf{E}$ and $\mathbf{E} \rightarrow \mathbf{R}$ reactions for thermodynamical reasons, but also that the two redox reactions in the reductive phase *within* the cycle (i.e. $\mathbf{H} \rightarrow \mathbf{E_H}$ and $\mathbf{E_H} \rightarrow \mathbf{R}$) are expected to have $E_{m,7}$ values considerably higher than those measured at equilibrium. Whether this must be the case for *both* haem a_3 and Cu_B depends on the still unresolved issue of whether both reactions of the reductive phase are equally coupled to proton translocation, as tentatively depicted in Fig. 2. Alternatively, both protons might be pumped in the step $\mathbf{H} \rightarrow \mathbf{E_H}$, in which case the notation $\mathbf{E_H}$ would be superfluous and equal to **E**. As discussed above, the recent experiments by Ruitenberg et al. [16] suggest proton translocation coupled to $\mathbf{E_H} \rightarrow \mathbf{R}$, but they do not provide definite proof for this possibility.

6. The structural difference between H and O

A higher $E_{m,7}$ for state **H** than for state **O** would be consistent with the idea that **H** decays spontaneously into **O** with loss of energy. The basis for this reaction may be stabilisation of the cupric form of Cu_B relative to the cuprous form, which could be due to binding of a ligand, such as OH^-, to Cu_B[II]. In recent experiments (see Refs. [22,23]), we have indeed found clear differences between the state of the recently oxidised binuclear site (**H**), as compared to its relaxed form (**O**). However, further work will be required to define the state **H** structurally, and to precisely assign how the reductive phase of the catalytic cycle is coupled to proton translocation. Finally, it will also be necessary to explore the possible relationship between this state and the previously described ''pulsed'' [24] and ''fast'' [25] forms of the oxidised enzyme.

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